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### **MALDI-TOF/TOF mass spectrometric assignment of Leu/Ile in PVK/CAP2b neuropeptides from single neurohemal organ preparations of four flies\*)**

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**Abstract:** MALDI-TOF/TOF tandem mass spectrometry has been applied to determine the complete sequences of the PVK/CAP2b neuropeptides in abdominal dorsal sheath tissue of the housefly *Musca domestica*, flesh fly *Neobellieria bullata*, stable fly *Stomoxys calcitrans* and horn fly *Haematobia irritans*. This peptidomic analysis of single neurohemal organ preparations allows, for the first time in arthropods, the unambiguous assignment of internal Leu/Ile positions not distinguishable by previous mass spectrometric techniques. The identification of these novel neuropeptides adds to our knowledge of the peptidomes of flies, and can aid in the development of neuropeptide-based control strategies of these insect pests.

**Keywords:** periviscerokinin, *Musca domestica*, *Neobellieria bullata*, *Stomoxys calcitrans*, *Haematobia irritans*, peptidomics, insects

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## INTRODUCTION

Neuropeptides are important messenger molecules that occur in a great variety of forms and are implicated in the regulation of critical physiological processes such as diuresis, digestion, development and reproduction in insects [1]. In the past several years, new developments in matrix-assisted laser desorption-time-of-flight mass spectrometry (MALDI-TOF MS) have afforded very sensitive *de novo* sequencing of peptides *via* direct analysis of single neurosecretory organs or nerves, including those of insects [2-4] *via* the post-source decay (PSD) technique. The technique alleviates the need for large numbers of specimens and the time-consuming and expensive efforts required to isolate and determine the primary structure of neuropeptides *via* traditional chromatographic and chemical sequencing techniques [5]. However, MS analysis of insect neuropeptides has failed in the past to distinguish between the isomers Leu and Ile, which have identical masses. These earlier studies were limited to low energy fragmentations of the ion of interest. A primary limitation of PSD peptide sequencing is that the internal energies of the  $[M + H]^+$  ions are not sufficient to yield the side chain cleavages necessary to distinguish Leu and Ile. However, recent innovations in MALDI-TOF MS have allowed analysis of high-energy collision-induced dissociation of the parent ions of peptides that reveal unique mass patterns for the sidechains of Leu and Ile [6, 7].

In this paper, we report on the use of MALDI-TOF/TOF tandem MS to undertake the first identifications of the sequences of PVK/CAP2b neuropeptides, including the unambiguous assignment of Leu/Ile positions, from single neurohemal organ preparations of adults of four species of flies; the housefly *Musca domestica*, flesh fly *Neobellieria bullata*, stable fly *Stomoxys calcitrans* and horn fly *Haematobia irritans*, the latter two representing important livestock pests. PVK/CAP2b's are typical of the abdominal neurohemal system of insects [8], usually stored in abdominal perisymphatic organs. The PVK/CAP2b class of neuropeptides has been shown to elicit both myotropic activity and stimulation of Malpighian tubule fluid secretion in insects [8], physiological processes critical to survival.

## MATERIALS AND METHODS

Insects were reared and dissected as previously described [2]. MALDI analysis was performed on the ABI proteomics analyzer (Applied Biosystems, Framingham, MA). Due to the nature of the neural tissue samples, all acquisitions

were taken in the manual mode. For tandem MS experiments, the acceleration was 1 kV in all cases. In order to change the net amount of collisions to the primary ions in the collision-induced dissociation (CID) experiments, the atmospheric air pressure was increased according to the three pressure settings available to the instrument (none, medium, high). The true pressure within the cell cannot be measured. The fragmentation patterns were evaluated by the Applied Biosystems Data Explorer software package.

## RESULTS AND DISCUSSION

PVK/CAP2b peptides of insects are typical of neurosecretory neurons in the abdominal ganglia and are accumulated in perisymphatic organs until release. Larval perisymphatic organs of cycloraphous Diptera, however, become incorporated into the dorsal ganglionic sheath [9] during the metamorphosis. The abdominal dorsal sheath, which was dissected in this investigation, therefore contains relatively large amounts of peptidergic neurohormones. Direct analysis of abdominal dorsal sheath tissues from adults of the housefly *Musca domestica*, flesh fly *Neobellieria bullata*, stable fly *Stomoxys calcitrans* and horn fly *Haematobia irritans* were conducted via MALDI-TOF/TOF MS. High energy CID of the PVK/CAP2b peptides reveal unique patterns for the sidechains of Leu and Ile [6, 7]. Fragments of native PVK/CAP2b's of the flies include a prominent 'w7a' fragment ion, indicative of Leu in these peptides. Indeed, the spectra of synthetic versions of the PVK/CAP2b peptides containing Leu taken under the same conditions were essentially identical. In contrast, if the PVK/CAP2b peptides contained Ile, the mass spectra under conditions of high gas would reveal a different mass for the 'w7a' fragment, along with two diagnostic satellite ions, a 'v-ion' and a 'wb-ion'. Thus, the PVK/CAP2b peptides of the four species of flies can be unambiguously assigned the sequences listed in Table 1. While the internal L/I pairs could be unambiguously assigned, tandem mass spectrometry cannot distinguish between Leu and Ile at the C-terminus, as is the case with Neobu-PVK-2. Nonetheless, the possible sequences for Neobu-PVK-2 have been narrowed from eight to two using the analysis of spectra from MALDI-TOF/TOF tandem mass spectrometry (Table 1).

**Table 1.** Amino acid sequences of PVK/CAP2b peptides native to four species of flies determined by MALDI-TOF-TOF tandem mass spectrometry compared with the fruit fly and a mosquito

| Species                | PVK/CAP2b-1              | PVK/CAP2b-2                        |
|------------------------|--------------------------|------------------------------------|
| <i>S. calcitrans</i>   | AGGASGL <u>L</u> YAFPRVa | NAK <u>L</u> YPVPRVa               |
| <i>H. irritans</i>     | AGGASGL <u>L</u> YAFPRVa | NAK <u>L</u> YPMPRVa               |
| <i>M. domestica</i>    | AGGTSG <u>L</u> YAFPRVa  | AS <u>L</u> FNAPRVa                |
| <i>N. bullata</i>      | NGGTSG <u>L</u> FAFPRVa  | AG <u>L</u> IVYPR[ <u>L/I</u> ]a * |
| <i>D. melanogaster</i> | GANMGL <u>L</u> YAFPRVa  | ASGLVAFPRVa [10]                   |
| <i>An. gambiae</i>     | GPTVGL <u>L</u> FAFPRVa  | QGLVPFPRVa [11]                    |

\*MALDI-TOF-TOF tandem MS cannot distinguish between Leu and Ile at a C-terminal position [7].

It is clear that the Leu at the position located 7 residues from the C-terminus in these four PVK/CAP2b sequences from stable fly and horn fly is conserved within and across species (Table 1). Leu at this specific position is typical of other PVK/CAP2b of insects that were sequenced by Edman degradation in earlier studies [8] or for which genes have already been published [10, 11]. In general, PVK/CAP2b sequences are well conserved throughout the 6 species of flies that have been studied to date (Table 1). The PVK/CAP2b-1 sequences of the housefly, stable fly and horn fly are identical, and the PVK/CAP2b-2 sequences of the stable fly and horn fly differ only by a single residue in the fourth position from the C-terminus (V vs M) (Table 1).

In summary, MALDI-TOF/TOF tandem MS has been used to identify the sequences for the PVK/CAP2b neuropeptides in the housefly, flesh fly, stable fly and horn fly *via* direct analysis of nerve tissue. For the first time in an arthropod, this analysis includes an unambiguous assignment of the internal, isotopic residues Leu vs Ile. The work adds to our knowledge of the peptidomes of flies; and the identification of the specific structures of the PVK/CAP2b neuropeptides, implicated in the regulation of diuretic and myotropic processes, may aid in the development of mimetic analogs capable of disrupting these critical physiological processes in pest flies.

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